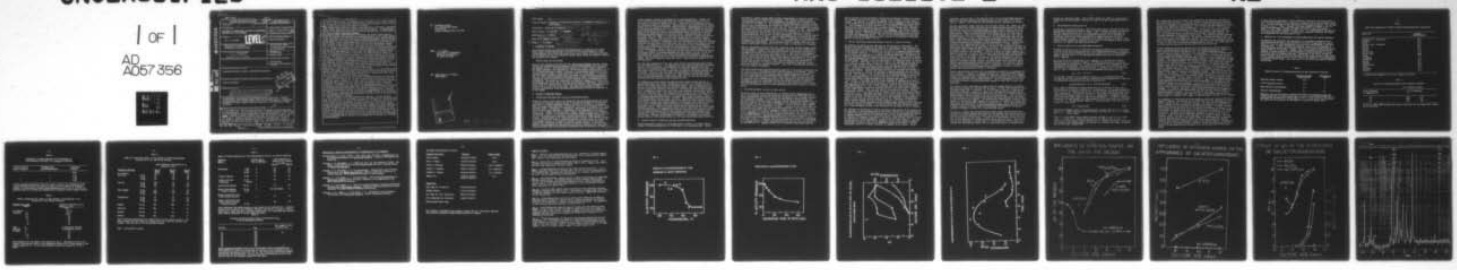
AD-A057 356 MINNESOTA UNIV ST PAUL DEPT OF BIOCHEMISTRY F/G 6/1
INFLUENCE OF CARBOHYDRATE RESIDUES ON FUNDAMENTAL PROPERTIES OF--ETC(U)
JUL 78 J E GANDER DAHC04-75-G-0179
UNCLASSIFIED ARO-13218.2-L NL

1 OF 1
AD A057 356



END
DATE FILMED
9-78
DDC

Unclassified
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: 13218.2-L	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): INFLUENCE OF CARBOHYDRATE RESIDUES ON FUNDAMENTAL PROPERTIES OF GLYCO-ENZYMES	5. TYPE OF REPORT & PERIOD COVERED: Final, 15 June 1975-14 June 1978
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Dr. J. E. Gander	8. CONTRACT OR GRANT NUMBER(s): DAAG29-75-G-0179
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of Minnesota, Minneapolis, Minnesota 55455	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 1T161102B71D Basic Research in Life Sciences
11. CONTROLLING OFFICE NAME AND ADDRESS: U. S. Army Research Office, Post Office Box 12211, Research Triangle Park, NC 27709	12. REPORT DATE: 10 July 1978
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report): unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

LEVEL II

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

same as statement

DDC PROCESSED AUG 9 1978 F

18. SUPPLEMENTARY NOTES

THE VIEW, OPINIONS, AND/OR FINDINGS CONTAINED IN THIS REPORT ARE THOSE OF THE AUTHOR(S) AND SHOULD NOT BE CONSTRUED AS AN OFFICIAL DEPARTMENT OF THE ARMY POSITION, POLICY, OR DECISION, UNLESS SO DESIGNATED BY OTHER DOCUMENTATION.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Glycoproteins, glyco-enzymes, polysaccharides, glycohydrolases, affinity chromatography, galactofuranosidase, fungi, Penicillium species, carbohydrate degradative enzymes, thermal stability, proteases, acid hydrolases, fungal nutrition,

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A. A procedure for isolation and purification of exo-β-D-galactofuranosidase has been developed. This procedure provides an enzyme preparation that is free of contaminating proteases, phosphatases, other glycohydrolases, and 5'-nucleotidase. Studies on thermal stability show that the enzyme is stable up to 55°C at pH 4. However, stability decreases with increasing pH. At pH 7.5 the enzyme is relatively unstable even at 4°C. Functional group analysis, combined with amino acid analysis, suggest that this decrease in stability does

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

78 08 07 179

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

not result from oxidation of sulfhydryl groups, but that it more likely results from an increase in carboxylate negative charges which probably serves to alter the secondary and tertiary structure of the enzyme.

The enzyme binds relatively tightly to dextrans and to cellulose, a property which could be exploited in its removal from natural sources. It appears that several of the other acid hydrolases (proteases, phosphatases, 5'nucleotidases) also bind to these carbohydrate polymers.

B. Extensive studies were conducted to determine the factors which serve to enhance or decrease the appearance of galactofuranosidase in the growth medium. It was shown that a series of substances (diammonium phosphate, sodium carbonate sodium hydroxide) all resulted in increased galactofuranosidase activity in the growth medium. Bovine serum albumin gave a 5-fold increase in galactofuranosidase activity in the growth medium The results of these experiments suggest that secretion of the enzyme occurs only after the pH of the medium is above 4 to 5. Bovine serum albumin may be providing protection of galactofuranosidase activity as well as increasing the pH. There is rapid loss of enzyme activity when the pH of the medium is 7.5 or greater. The addition of substances which result in extending the number of days that the growth medium is below pH 4 serve to decrease the onset of appearance of galactofuranosidase in the culture filtrate. The addition of 2-deoxy-D-glucose and bovine serum albumin resulted in a marked, although not complete, inhibition of synthesis/secretion of galactofuranosidase activity even though the pH of the culture filtrate was 6.2. It was found also that the appearance of and relative activities of other acid hydrolases approximately paralleled that found for galactofuranosidase. We conclude that the signal for synthesis and release of lytic enzymes by this Penicillium species is monitored by a pH sensitive mechanism.

These data suggest that the release of lytic enzymes can be controlled by the addition of substances which buffer the pH between 3 and 4 and may be of practical significance in decreasing fungal destruction of clothing and other items which are subject to attack by these organisms.

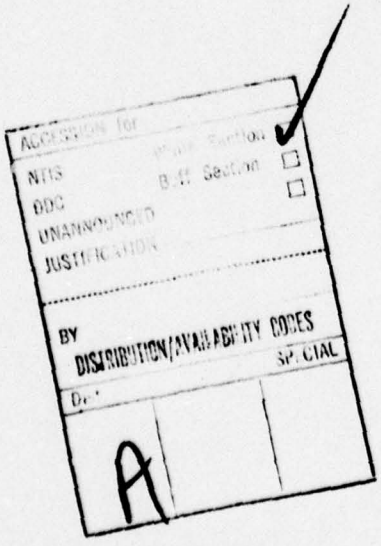
C. Galactofuranosidase may be considerably more widespread than was originally presumed as it was found in all species of Penicillium tested and also in one Aspergillus species tested. As a result, galactofuranosyl-containing heteropolysaccharides and glycoproteins may also be more widespread than is currently recognized. For example, we have shown that Penicillium varians secretes a glycopeptide containing galactopyranosyl and glucopyranosyl residues when grown in the modified Raulin-Thom medium. The culture filtrate has at least 5-fold more galactofuranosidase activity than found in P. charlesii media. However, the addition of 2-deoxy-D-glucose to the growth medium results in the formation of a glycopeptide containing galactofuranosyl residues as well as galactopyranosyl and glucopyranosyl residues.

D. Physical studies on the glycopeptide were conducted. We have evidence suggesting that there is considerable glycopeptide-glycopeptide interaction and that this interaction is enhanced by calcium ions. In addition, the shortening of the number of galactofuranosyl residues per galactan chain results in an increase interaction between phosphoethanolamine residues and solvent (water). ^{13}C-Nuclear magnetic resonance studies have been conducted to obtain additional evidence on the structure of the glycopeptide by a method independent of that used previously. We have been able to confirm the presence of galactofuranosyl residues in the polymer and the presence of α-1,2 mannopyranosyl linkages. This work is continuing with an objective of establishing the position of attachment of the phosphoethanolamine residues to the polymer and of phospho-N,N'-dimethylaminoethanolamine.

TO: Commanding Officer
U.S. Army Research Office
PO Box 12211
Research Triangle Park, NC 27700

FROM: J. E. Gander
Department of Biochemistry
University of Minnesota
St. Paul, MN 55108

RE: ARO Project No. P-13218-L
Final Report



Final Report (6)

Title of Project: (6) Influence of Carbohydrate Residues on Fundamental Properties of Glyco-enzyme

Institution: University of Minnesota (9) Final rept.

Report Period: 16 June 1975 - 15 June 1978,

ARO Project No. P-13218-L (11) 10 Jul 78

Grant No. (15) DAHC04-75-G-0179 (14) ARO

(10) Author: J. E. Gander (19) 13218.2-L (12) 26 p.

(16) 1T161102B71D

1. Statement of Problem:

This research project was directed toward determining the properties of a glyco-enzyme (exo-β-D-galactofuranosidase) secreted into the growth medium by a Penicillium species, which caused the enzyme to have great thermal stability at ambient (25°C) temperatures and to determine the factors which control the combined synthesis and secretion of the enzyme.

Initial status of the problem.

Prior to this research we had observed that Penicillium charlesii secretes a unique glycopeptide (peptidophosphogalactomannan) into its growth medium after the medium was depleted of NH_4^+. This glycopeptide contains ten galactan chains attached to the mannan backbone of the glycopeptide. The galactan chains contained from 2 to 25 5-O-β-D-galactofuranosyl residues, resulting in a glycopeptide preparation with an apparent molecular weight ranging from about 70,000 to 25,000 g/mole. It was found that the fungus secreted an enzyme which hydrolyzes the galactofuranosyl residues from the 5-O-β-D-galactofuranosyl-containing galactan chains of peptidophosphogalactomannan, and that the action of this enzyme accounted for the heterogeneity in the glycopeptide. The enzyme also hydrolyzes the glycosidic linkage of 1-O-methyl-β-D-galactofuranoside but not any of the other three methyl-D-galactosides. The optimum pH was found to be approximately 4 and unlike most enzymes, this glycohydrolase was stable at 25°C at pH 4 for several days. The enzyme was partially purified by affinity chromatography using peptidophosphogalactomannan attached to Sepharose 4B as the ligand.

2. Summary of Important Results

A. Purification and properties of exo-β-D-galactofuranosidase

During the course of this work it was found that the galactofuranosidase also binds to dextran or cellulose, polysaccharides whose glycosyl residues are structurally unrelated to β-D-galactofuranosyl residues, and that this binding is pH dependent. Thus, the enzyme binds essentially irreversibly at pH 4 and is released at pH 7 with phosphate buffer. Phosphatase, β-glucosidase, and protease activity was also bound and released by these conditions. Examination of the homogeneity of the enzyme eluted at pH 7 showed that this procedure effected minimal purification of galactofuranosidase. However, it did show that a number of fungal enzymes bind to dextrans and that the dextrans are potentially valuable adsorbents of fungal degradative enzymes. Chromatography of the enzyme preparation on BioGel P-100 resulted in about a 2-fold purification; however, galactofuranosidase activity eluted in a broad band with maximum activity in the apparent molecular weight region of 50,000 g/mole. Finding other degradative enzymes, especially protease activity, in even the most highly purified preparations obtained from the affinity column and the other purification procedures used was particularly disturbing since the activities of these not

enzymes probably resulted in heterogeneity in galactofuranosidase. However, protease activity was not measurable until the pH was increased above 4.5 and all operations were routinely conducted at pH 4. Purification was attempted by using the following sequence of operations: Precipitation of the proteins from the growth medium with a 65% saturated ammonium sulfate solution, chromatography of the resolubilized proteins applied at pH 5.5 to a DEAE-cellulose column followed by obtaining galactofuranosidase released by 0.1 M phosphate buffer and concluding by rechromatography of the protein(s) eluting with galactofuranosidase activity on the peptidophosphogalactomannan-Sepharose 4B affinity column. Galactofuranosidase activity was eluted from this column as a sharp band of activity with 1-O-methyl-β-D-galactofuranoside (table 1). This series of steps removed protease activity; however, phosphatase, β-D-glucosidase activity and 5'-nucleotidase activity were found as contaminating activities. Furthermore, disc gel electrophoresis showed that although there were two major bands of protein there were also several minor bands of protein visible. We attempted to obtain further purification by using a peptidophosphomannan-Sepharose 4B affinity column (the galactofuranosyl residues were removed with galactofuranosidase). However, none of the contaminating enzymes bound to this ligand. We conclude from these investigations that either the contaminating enzymes are binding tightly to galactofuranosidase and thus copurify with it, or that the galactofuranosyl residues on the ligand (peptidophosphogalactomannan) serve as recognition and binding sites for a series of proteins in addition to galactofuranosidase. The latter suggestion seems more likely. Experiments are underway to test both hypotheses.

The amino acid composition of our most highly purified galactofuranosidase preparation was obtained (table 2). We recognize that these data represent only an approximation to the correct amino acid composition of galactofuranosidase due to the presence of contaminating enzymes. However, the relative composition of acidic and basic amino acids in all of the proteins that co-purify by the above procedure may be similar. The table shows that there are approximately 90 potentially acidic amino acyl residues and only 17 basic ones. This suggests that the isoelectric point of galactofuranosidase should be relatively low. It was found by isoelectric focussing that the isoelectric point occurred at pH 4.0.

The influence of various functional group specific reagents on the activity of galactofuranosidase were tested. Enzyme was reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) which reacts with free sulfhydryl groups. This treatment caused no decrease in galactofuranosidase activity which suggests that free sulfhydryl groups are not required for activity of the enzyme. The enzyme was reacted with iodoacetate, an alkylating reagent that reacts only with histidinyl and methionyl residues under the conditions used. This treatment did not result in loss in enzyme activity. In contrast, when the enzyme was treated with glycine methyl ester and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, a procedure which amidates carboxyl groups, no galactofuranosidase activity could be detected. Appropriate controls showed that the galactose oxidase and peroxidase used in the assay were only slightly inhibited. These results suggest that carboxyl group(s) are important in the catalytic process or at least in maintaining charge distribution and conformation of the enzyme. Experiments will be conducted in which taurine replaces glycine methyl ester in the amidation reaction. These experiments should serve to show whether the inactivation of galactofuranosidase by amidation with glycine methyl ester was caused by alteration in charge distribution or in a more specific reaction since amidation with taurine will result in a modified galactofuranosidase containing the same charge distribution. Whatever the mechanism of catalysis, it is apparent that optimum enzymic activity of the protein occurs in a pH region approaching charge neutrality.

B. Thermal stability of partially purified galactofuranosidase

Galactofuranosidase activity in the growth medium is stable in 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 0.1 M EDTA and 0.1 mM o-phenanthroline. Galacto-

furanosidase purified through DEAE cellulose chromatography is much less stable than that purified only by affinity chromatography. That purified by DEAE cellulose chromatography contains very little carbohydrate and appears to be unstable at pH 7.5 even at -10°C (Table 3). The stability of galactofuranosidase purified through DEAE cellulose chromatography was examined by holding the enzyme preparation at pH 4 and the indicated temperature for 5 min. (Fig. 1). The enzyme retained full activity up through 55°C and the activity decreased rapidly between 60 to 70°C. Approximately 20% of the activity was retained even at 90°C. The influence of time at 60°C on galactofuranosidase activity (Fig. 2) shows that a rapid decrease in activity occurred during the first 30 min. and that approximately 40% of the original activity remained after 60 min. at 60°C. Methyl-β-D-galactofuranoside was used as the substrate in the assays for enzyme activity. Thus it is apparent that although galactofuranosidase is quite unstable at pH 7.5 it is reasonably stable at pH 4 even to relatively high temperatures. The instability at pH 7.5 and the stability at pH 4 suggests that enzyme activity may be lost as a result of oxidation of a -SH group.

However, experiments described above with sulfhydryl group specific reagents showed that the enzyme is insensitive to such reagents. The loss in activity may result from proteolysis. However, this also seems unlikely as proteolytic degradation of azoalbumin was minimal at pH 7 and maximal at pH 5. These results when combined with the amino acid composition data suggest that at pH's above 6 the observed rapid thermal inactivation may result from a large negative charge density on the enzyme which serves to cause a loss in secondary and tertiary structure of the enzyme due to repulsion of negative charges. Such a protein would be very susceptible to thermal inactivation. The 22 prolyl residues are undoubtedly important in determining the primary and secondary structure. Their role in maintaining stability at this time cannot be determined.

This enzyme preparation contained negligible quantity of associated carbohydrate and its role, if any, in determining enzyme stability appears to be minimal. However, the associated polysaccharide which is found in galactofuranosidase obtained by affinity chromatography on peptidophosphogalactomannan-Sepharose 4B may be to protect the enzyme from degradative enzymes rather than to alter thermal stability. This question can be resolved only when we have sufficient of the associated polysaccharide to carry out experiments in which the thermal stability of purified galactofuranosidase at various pH's and temperature are compared to those in which both galactofuranosidase and the associated polysaccharide are treated in a similar manner.

C. Galactofuranosidase activity in other species

During the course of these investigations we examined 8 other Penicillium species for a galactofuranosyl-containing exocellular glycopeptide and found that all produced a glycopeptide and that all except the glycopeptide from P. varians contained galactofuranosyl residues. We confirmed the observation of Haworth et al. that the exocellular glycopeptide from P. varians contains glycosyl and galactosyl but no mannosyl residues. We also found ethanolamine in the glycopeptide from P. varians. The galactosyl residues were stable to treatment with 0.01 N HCl at 100°C, conditions which release galactofuranosyl residues. Galactose was not released when the glycopeptide was treated with exo-β-D-galactofuranosidase. The amino acid composition of the glycopeptide from P. varians was similar to, but not identical to that from P. charlesii. We examined the culture filtrates from 14-day cultures of each of the 3 Penicillium species for exo-β-D-galactofuranosidase activity and found that all contained activity. Penicillium varians culture filtrates contained 3- to 5-fold greater galactofuranosidase activity than culture filtrates from P. charlesii. From these results it is apparent that galactofuranosidase activity is great enough in P. varians cultures to remove any galactofuranosyl moieties that are attached to the glycopeptide of P. varians assuming that the galactan chains branch from the

glycan backbone and that nonreducing terminal galactofuranosyl residues are exposed. This proposal was tested by growing P. varians in the presence of 15 mM 2-deoxy-D-glucose. The addition of 2-deoxy-D-glucose to 3-day P. varians cultures resulted in the formation of a glycopeptide which contained galactofuranosyl residues (Table 4). This glycopeptide is being characterized. Preliminary data have shown that acetolysis of the glycopeptide gives an oligosaccharide "finger print" completely different than that from the glycopeptide of P. charlesii. The results of these experiments clearly demonstrate that exo-β-D-galactofuranosidase is not limited to one fungal species. Galactofuranosidase activity has been demonstrated in culture filtrates from Aspergillus species. The role of the galactofuranosyl-containing galactan chains is of considerable interest relative to the function of the glycopeptide in the species where it occurs.

D. Factors influencing galactofuranosidase activity in culture filtrates

The influence of adding various substances to the culture was investigated. Preliminary experiments showed that the addition of $(NH_4)_2HPO_4$ to 6-day cultures enhanced the activity of galactofuranosidase in 14-day cultures. This increased galactofuranosidase activity was dependent on the quantity of ammonium phosphate added to the cultures (Table 5). A 3- to 4-fold increase in galactofuranosidase activity was obtained by the addition of 5.3 mmoles of ammonium phosphate. The addition of NH_4Cl or $(NH_4)_2SO_4$ inhibited galactofuranosidase synthesis. Substitution of Na_2HPO_4 for ammonium phosphate had no effect on galactofuranosidase secreted into the extracellular medium. Phosphate raises the pH because of its buffering effect in contrast to Cl^- and SO_4^{--} which lower the pH of the medium. The ammonium ion may also serve as a precursor of amino groups in proteins. The influence of ammonium phosphate, added to 6-day cultures, on the pH of the culture was determined and is shown in Fig. 3. Cultures containing added ammonium phosphate were at least 2 pH units higher than controls. Galactofuranosidase activity also increased more rapidly in the culture filtrate. However, the addition of ammonium phosphate did not seem to have any influence on the onset of appearance of galactofuranosidase in the culture filtrate. The influence of the time of addition of ammonium phosphate on the activity of galactofuranosidase in 14-day culture filtrates (Fig. 4) shows that the maximum enhancement by ammonium phosphate was obtained when added to 3-, 4-, 5- or 6-day cultures. The culture initially becomes depleted of ammonium ion at about 3 days. In contrast the addition of D-glucose, which caused some repression in galactofuranosidase activity in the culture filtrate, seemed to have little influence when added before day 8. This is understandable since the growth medium normally becomes approximately depleted of D-glucose between 8 and 9 days after transferring spores to the culture. Thus, the data could be interpreted as follows: Galactofuranosidase activity is released into the medium at some time following an increase in pH above 4 to 5. Thus those substances such as casein amino acids, bovine serum albumin and ammonium carbonate when added to the growth medium at day 3 result in an increase in pH (Fig. 5a) and they also result in the appearance of galactofuranosidase activity in 8-day culture filtrates (Fig. 5b). Similarly, the addition of NaOH or sodium carbonate to 3-day cultures resulted in an increase in pH of the culture filtrate and the appearance of galactofuranosidase starting at day-6 followed by a rapid increase in activity. Activity in culture filtrates decreased rapidly after 8 days presumably because of the instability of the enzyme at pH 8 or greater.

The influence of the addition of various potential carbon sources to the cultures on the appearance of galactofuranosidase in the culture filtrate was investigated prior to finding that the enhanced galactofuranosidase activity resulting from the addition of ammonium phosphate was due to increasing the pH of the growth medium above 4. The addition of D-mannose, D-galactose or sucrose to 6-day cultures served to inhibit the appearance of galactofuranosidase in the culture filtrates (table 5). The indicated quantities of cell wall from P. charlesii, dextran, yeast mannan on galactofuranosidase activities (table 5) all appeared to cause some decrease in galactofuranosidase activity in culture filtrates, as compared to the control. In one

experiment, shown in table 6, the addition of 50 or 75 mg of peptidophosphogalactomannan to each flask enhanced galactofuranosidase activity found in the culture filtrate. However, this result was not obtained when the experiment was repeated.

Previous experiments (Gander and Fang (1974) Biochem. Biophys. Res. Commun. 58, 368-374) have shown that 2-deoxy-D-glucose inhibits synthesis and secretion of galactofuranosidase into the extracellular medium, but that 2-deoxy-D-glucose had little effect on the synthesis of peptidophosphogalactomannan and its secretion into the growth medium. These investigations have been repeated and we have shown that D-glucosamine also inhibits the appearance of galactofuranosidase into the culture filtrate (Table 7), as does 2-deoxy-D-glucose. We have shown above (Fig. 5b) that bovine serum albumin serves to enhance galactofuranosidase activity several fold. Table 7 shows that the combination of bovine serum albumin and 2-deoxy-D-glucose resulted in depressed but measurable, galactofuranosidase activity. Further investigation of this phenomenon showed that the pH of cultures to which 2-deoxy-D-glucose had been added, did not increase as rapidly as that of controls or those to which bovine serum albumin was added (data not shown). An additional experiment was performed in which a 3-day culture was adjusted to pH 4.7 with NaOH on day 3 and 15 mM 2-deoxy-D-glucose was also added. A control to which no 2-deoxy-D-glucose was added was also used. The pH of 7-day culture containing the added 2-deoxy-D-glucose was 6.2 and that of the control was 6.5. There was no detectable galactofuranosidase activity in the former. In contrast, the control contained 0.17 units of activity per 50 µl of culture filtrate. We conclude that 2-deoxy-D-glucose both depresses the normal increase in pH of the growth medium and in some other manner represses galactofuranosidase synthesis and/or secretion. This provides indirect evidence that galactofuranosidase is either a glycoprotein or that in the absence of synthesis of the associated protective polysaccharide as caused by 2-deoxy-D-glucose, galactofuranosidase activity is destroyed.

Similar investigations on other lytic acid hydrolases released by P. charlesii have been initiated. In general, β-D-glucopyranosidase (1-O-(p-nitro-phenyl)-β-D-glucoside as substrate), protease (azoalbumin as substrate), phosphatase (p-nitrophenyl phosphate as substrate) and 5'-nucleotidase (RNA as substrate) activities increased or decreased parallel with galactofuranosidase. Thus the phenomenon of the influence of pH on release of galactofuranosidase appears to carry over to other acid hydrolases.

The results of these investigations suggest that this fungus monitors its requirement for lytic enzymes by a pH sensitive mechanism. We have shown previously (Klatt and Gander (1968) Can. J. Microbiol. 14, 579-585) that L-tartrate is taken up and metabolized only after D-glucose is essentially exhausted from the growth medium. When the Penicillium is grown under conditions where there is no organic acid in the growth medium it releases organic acids into the growth medium. It is of interest to know if these are re-assimilated and provide the signal (pH greater than about 4) for the release of lytic enzymes. This mechanism for signaling the release of lytic enzymes is a relatively simple control for the release of a battery of enzymes which then attack the extracellular substrates available to it. One of these substrates, produced by the Penicillium, is peptidophosphogalactomannan which provides at least a minimal quantity of energy source for maintaining the organism as its mycelia continue to elongate in search of a richer nutrient source.

The data also suggest that non-metabolizable weakly acidic salts might provide a practical means of preventing the release of lytic enzymes which are destructive of clothing and natural polymers with which these enzymes come into contact when they are released from the Penicillium. It should be of considerable interest to determine if this phenomenon is widespread among several genera of fungi. Possibly of even greater importance in current times, is the observation that the fungi can be tricked into overproducing enzymes that are potentially useful in degrading waste

products of biological origin. Most of these enzymes are stable to a wide range of temperatures and environmental conditions as long as the pH is maintained between 4 and 5.

E. Galactofuranosyl-binding proteins

In addition to galactofuranosidase, three other major species of protein bind to the glycopeptide covalently attached to agarose. These "binding" proteins are released from the ligand with dilute salt in acetate buffer. We now have shown that the "binding" proteins do not bind to the glycopeptide-Sepharose if the adsorbent is extensively treated with galactofuranosidase to remove essentially all of the galactofuranosyl residues. The function of the binding proteins is unknown. Their binding to the glycopeptide-sepharose may be coupled to the binding of galactofuranosidase and its tightly associated polysaccharide.

F. Physical properties of peptidophosphogalactomannan

Currently relatively little is known about the physical properties or conformational structure of the polysaccharide portion of peptidophosphogalactomannan. Investigations of polysaccharide conformation have been conducted on only a limited number of polymers and none similar to the phosphogalactomannan region of the glycopeptide from P. charlesii.

The viscosity of the glycopeptide from P. charlesii was measured in a Cannon-Ubbelonde capillary dilution viscometer. The drop time was measured at varying concentrations of glycopeptide. The density was measured with a picnometer at each glycopeptide concentration. The specific viscosity [η_{sp}] was calculated at each concentration of glycopeptide from the density, ρ, and drop time, t, for pure solvent (ρ' and t', respectively) and for the glycopeptide using equation [(1)].

$$\eta_{sp} = (\rho t - \rho' t')/\rho' t' \qquad [(1)]$$

The intrinsic viscosity [η] and Huggins constant, k, are determined from data relating the specific viscosity per unit concentration of glycopeptide as a function of glycopeptide concentration according to equation [(2)].

$$\eta_{sp}/[\text{glycopeptide}] = [\eta] + k[\eta]^2[\text{glycopeptide}] \qquad [(2)]$$

Over a range of glycopeptide concentrations from 120 to 600 mg dl^{-1} there was a linear decrease in specific viscosity mg^{-1} of glycopeptide. An intrinsic viscosity of 0.156 dl g^{-1} and a Huggins constant of 9.06 was calculated from the intercept and slope respectively. The intrinsic viscosity is much larger than observed for hemoglobin (68,000 daltons) which is 0.036 dl g^{-1}. Hemoglobin is a globular molecule with a molecular weight similar to that of the glycopeptide. In contrast, cellulose tricaprylate (70,000 daltons) in γ-phenylpropanol at 0°C. has an intrinsic viscosity of 0.38 dl g^{-1}. The hydrodynamic radius, R_e, is proportional to the intrinsic viscosity (equation [(3)]).

$$[\eta] = (\pi N_{av}/30\mu)R_e^3 \qquad [(3)]$$

Thus the hydrodynamic radius of the glycopeptide is larger than that of a globular protein of a similar molecular weight but smaller than that of a random coil polymer of similar molecular weight.

Solid, uncharged spheres have a Huggins constant of about 2, and that for random coils is somewhat less. A Huggins constant of 9.06 suggests that the specific viscosity per unit concentration of glycopeptide is strongly concentration dependent. This suggests that polymer-polymer interactions are occurring even though the polymer was in 0.2 M ionic strength NaCl.

The translational diffusion constant was measured from the Rayleigh linewidths of laser light scattered from a 22.5 mg ml^{-1} solution of glycopeptide and by measuring the rate of spreading of the boundary between the solution of glycopeptide and the solvent in an analytical ultracentrifuge. The diffusion constant (corrected to 20°C) obtained from light scattering technique was 1.2×10^{-7} cm^2 sec^{-1}. This technique provides a weight averaged diffusion constant. The boundary spreading technique gave a value of 1.065×10^{-6} cm^2 sec^{-1} at infinite dilution of glycopeptide. However, the diffusion constant as measured by the boundary spreading technique was extremely dependent on glycopeptide concentration. The diffusion constant increased 0.150 cm^2 sec^{-1} mg^{-1} of glycopeptide. The boundary spreading technique weights the species more heavily that diffuse more rapidly. This may in part account for a portion of the difference in diffusion coefficient obtained by the two methods especially if there is some type of association between polymeric species. This association is increased upon the addition of divalent metal ions such as calcium.

We have conducted preliminary examination of the fluorescence emission spectra of dansyl-peptidophosphogalactomannan and dansyl-phosphogalactomannan as a function of their position of elution from BioGel P-60. N,N'-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl) derivatizes the ethanolamine, lysine and N-terminal amino acid(s) of peptidophosphogalactomannan. The polypeptide is released from the phosphogalactomannan by treatment with NaOH which results in an α,β-elimination type of reaction. The reisolated phosphogalactomannan contains a dansyl-ethanolamine residue attached to the mannan backbone. The derivatized polymers were fractionated and the spectra of the fractions related to the fraction number. The fluorescence emission maximum, E_{max}, when activated at 344 nm, is a function of the apparent molecular weight as shown by gel filtration chromatography (Table 8). We have shown previously that the apparent molecular weight as determined by sedimentation equilibrium ultracentrifugation is a function of the percentage of galactosyl residues and is independent of the number of mannosyl and phosphoryl residues. The galactan chains may contain, on the average, as many as 25 galactofuranosyl residues or as few as 1 to 2 which results in glycopeptide with molecular weight extremes of 70,000 and 25,000 g $mole^{-1}$, respectively. The E_{max} shows a red shift as the apparent molecular weight of dansyl-phosphogalactomannan decreases (Table 8). E_{max} of dansyl-phosphogalactomannan approaches 540 nm observed with dansyl-ethanolamine, as the number of galactofuranosyl residues per chain decreases. A 20 nm red-shift was obtained from the largest to smallest dansyl-phosphogalactomannan species measured, and the increase in E_{max} from fraction to fraction was essentially constant over the 40 fractions examined. In a separate experiment, dansyl-phosphogalactomannan with an E_{max} of 500 nm was treated with galactofuranosidase and the partially degraded dansyl-phosphogalactomannan reisolated. The E_{max} was measured and found to be at 520 nm. These experiments strongly suggest that the E_{max} of fluorescence emission of the dansyl group is a function of the number of galactofuranosyl residues in the polymer.

The dependence of fluorescence emission maximum on solvent has been described for a number of solvents. The transfer of the fluorescent dye from a nonpolar to a polar solvent usually results in a red-shift in E_{max}. The basis for this dependence of E_{max} on solvent was first proposed by Lippert as the influence of solvent on the interaction with the excited state of the fluorescent probe. If solvent molecules relax around the excited state prior to emission of the photon, the energy of the excited state is lowered which results in the emission of a photon of lower energy, a red-shift. Ainsworth and Flanagan have suggested that the presence of a polar residue is a necessary but not sufficient condition to produce a red-shift in emission. Thus, a red-shift is obtained only if the dipole is mobile. The red-shift in emission observed as the average number of galactofuranosyl residues per galactan chain decreased likely reflects the interaction between the galactan chain(s) and the dansyl-ethanolamine residue in the mannan backbone. Currently it is not known if the galactan chain(s) influence solvent interaction with the dansyl group or if the chains restrict the mobility of the excited state of the dansyl group.

We have initiated experiments to obtain additional information and understanding of the structural features of the glycopeptide through the use of spectroscopic techniques. We have performed ^{13}C nmr spectroscopy on phosphogalactomannan. The spectrum is shown in Fig. 7. Peaks 1 through 5 have been assigned to carbons of the 1,5-linked galactofuranosyl residues previously described by Gorin and Mazurek. Peaks 6, 7 and 8 likely represent C-1 from α-1,6, α-1,2 mannopyranosyl residues and β-1,3-galactofuranosyl residues. Further work is underway to identify the carbon atom responsible for each peak shown in Fig. 7.

G. Synthesis of 1-O-(p-nitrophenyl)-β-D-galactofuranoside and related substances

Considerable time and effort has been devoted to the chemical synthesis of this galactofuranoside because it would decrease by an order of magnitude the time required to assay for galactofuranosidase activity and it would provide a much more sensitive assay. We have succeeded in preparing 1-bromo-2,3,5,6-tetraacetyl-D-galactofuranoside anomers and to convert these to the O-methyl-D-galactofuranosides using methanol as the acceptor. However, when p-nitrophenol was substituted for methanol a minimal quantity of O-(p-nitrophenyl)-D-galactofuranosides was formed even though very dry reagents were used. Replacement of the acetyl residues by benzoyl residues served to make the intermediate more stable and gave larger yields of the O-methyl-D-galactoside but did not improve the quantity of O-(p-nitrophenyl)-D-galactofuranoside formed. Thus, this aspect of the project has been particularly unrewarding.

Table 1

Specific Activity of Galactofuranosidase in Each Purification Fraction

	Specific Activity μmoles/min·mg	Purification -fold
Dialyzed culture filtrate	0.15	1
40-70% $(NH_4)_2SO_4$ fraction	0.16	1
DEAE cellulose chromatography	1.4	9
Affinity chromatography	5.7	38

Routinely, the reaction mixture contained 600 nmoles β-D-methylgalactoside and various quantities of enzyme preparations in 0.2 ml of 66 mM acetate buffer pH 4.0. Reactions were incubated for varying times at 37°C. At the end of the incubation the amount of free galactose was determined in a galactose oxidase assay.

Table 2

Amino Acid Composition of Highly Purified Galactofuranosidase Preparation

Amino Acid	n moles / n mole protein[a]
Aspartic Acid + asparagine	47.8
Threonine	34.2
Serine	42.4
Glutamic acid + glutamine	41.5
Proline	22.1
Glycine	40.7
Alanine	38.4
Half-cystine	1.4
Cysteic Acid	None
Valine	21.0
Methionine	7.1
Isoleucine	15.6
Leucine	30.8
Tyrosine	16.3
Phenylalanine	16.3
Lysine	14.6
Histidine	7.0
Arginine	10.0

a Analysis was conducted on 90 µg (2.1 nmoles) of protein

Table 3

Activity of Galactofuranosidase After Indicated Treatment

24 hr incubation at temperature °C	% of original activity: pH of incubation 4	7
25°	96%	12%
4°	100%	74%
-10°	94%	71%

50 µl of a pooled DEAE fraction were used in each assay and the samples were assayed at 37° for 2 hrs.

Table 4

Influence of 2-Deoxy-D-glucose on the Occurrence of Galactofuranosyl Residues in P. varians Glycopeptide

2-Deoxy-D-glucose added to culture	Treatment with galactofuranosidase	D-galactose released
		µmole
-	-	0.05
-	+	0.12
+	-	0.03
+	+	0.85

3 mg of glycopeptide obtained from 6-day cultures grown in the presence of 15 mM 2-deoxy-D-glucose added to the culture on day 3 or in the absence of 2-deoxy-D-glucose was treated with or without galactofuranosidase for 12 hr at 37°C and the galactose which was released was measured with a galactose oxidase coupled reaction.

Table 5

Effect of Ammonium Salt Added to 6-Day Cultures on Galactofuranosidase Activity in 14-Day Culture Filtrates

Ammonium Salt Added	mmoles	Galactose released by 50 µl medium in 2 hrs. µmole
no addition		.08-.11
$(NH_4)_2PO_4$	7.5	>0.25
	5.0	>0.25
	3.0	.11
	1.0	.08
	.3	.03
	.1	.03
	.01	.03
NH_4Cl	14	no detectable activity
$(NH_4)_2SO_4$	7.5	no detectable activity
$Na\ H_2PO_4$	6	.12
	3	.12
	1.5	.12
	.75	.12
	.3	.12

Solid ammonium salt was added to the cultures at day 6. Routinely at day 14 1 ml aliquot of medium was filtered, was subsequently dialyzed for 24 hours against 66 mM acetate buffer pH 4.0. 50 µl of the dialyzed filtrate was assayed at 37° for 2 hours.

Table 6

Effect of Saccharides Added to 3-Day Culture on Galactofuranosidase Activity in 14, 16, and 20 Day Cultures

Saccharide Addition		µmoles Galactose released by 50 µl medium in 2 hrs. Day 14 µmole	Day 16 µmole	Day 20 µmole
no additions		.08-.11	.08	.12
Cell wall	25 mg	.07	.07	.10
	50 mg	.02	.03	.09
	75 mg	.02	.03	.05
dextran	25 mg	NDA*	.03	.05
	50 mg	.04	.07	.14
	75 mg	.02	.02	.05
yeast mannan	25 mg	.03	.03	.07
	50 mg	NDA	.02	.03
	75 mg	.05	.03	.04
glycopeptide	25 mg	.02	.05	.11
	50 mg	.11	.15	.18
	75 mg	.20	.16	.24
mannose	750 mg	NDA	NDA	.03
galactose	750 mg	NDA	.01	.07
glucose	750 mg	.02	.05	.11
sucrose	750 mg	NDA	NDA	.02

Solid saccharide preparations were added directly to the culture at day 3. Aliquots, removed from the culture on varying days, were filtered, dialyzed, and assayed. Cell wall was 2 1/2-3 day purified samples.

*NDA - no detectable activity.

Table 7

Effect of Various Substances on Galactofuranosidase Activity in Culture Filtrates

Substance Added		Culture Age at Time of Addition	µmole Galactose released by 50 µl medium in 2 hrs	
			Day 14	Day 18
Glucosamine	3 mM	3	.04	.20
	6 mM	3	.06	.05
	10 mM	3	.03	.04
2-Deoxy-D-Glucose	15 mM	6	.03	.04
2-Deoxy-D-Glucose + $(NH_4)_2HPO_4$	15 mM 5 mmoles	6	.03	.03
Bovine Serum Albumin	750 mg	6	.52	.56
Bovine Serum Albumin + 2-Deoxy-D-Glucose	750 mg 15 mM	6	not determined	.12
Ethanol precipitate from 6-Day culture filtrate		6	.15	.21
Ethanol precipitate from 6-Day culture filtrate + 2-Deoxy-D-Glucose	15 mM	6	.04	.04

Solid substances were added directly to the culture on the indicated day. Aliquots, removed from the culture were filtered, dialyzed, and assayed. Controls of cultures containing no added substances contained approximately 0.14 µmole galactose released under similar conditions in 14-day culture filtrates.

Table 8

Influence of Galactan Chain Length on Fluorescence E_{max} of Dansyl-phosphogalactomannan

Fraction	E_{max}	Ave. number of gal_f residues/chain
	nm	
38	500	20
42	503	
46	507	
54	509	
59	512	
64	516	
69	519	
74	520	2

Dansyl-phosphogalactomannan from 10 mg of heterogenous dansyl-glycopeptide was fractionated on a BioGel P-60 column and the spectrum of the fluorescence emission was recorded following activation at 344 nm. The fluorescence spectrum for dansyl-ethanolamine was recorded also. The E_{max} for dansyl-ethanolamine was 539 nm and that for each of the indicated samples is shown above.

Publications, Abstracts and Manuscripts in Preparation or to be Prepared

Rietschel-Berst, M. et al. (1977) J. Biol. Chem. 252, 3219-3226. Extracellular exo-β-D-Galactofuranosidase from Penicillium charlesii. Isolation, Purification and Properties.

Pletcher, C. H. and Gander, J. E. (1978) Fed. Proc. 37, 1525 (Abstract # 1411). The Effect of Nutrients on exo-β-D-Galactofuranosidase Activity in Culture Medium of Penicillium charlesii.

Pletcher, C. H. and Gander, J. E. (in preparation). Extracellular exo-β-D-Galactofuranosidase from Penicillium charlesii. Effect of Nutrients on Activity in Culture Filtrate. (To be submitted to Journal of Bacteriology).

Unkefer, C. J. and Gander, J. E. (in preparation). Exo-β-D-Galactofuranosidase in Penicillium varians Culture Filtrates. Influence on the Composition of the Major Exocellular Glycopeptide.

Unkefer, C. J. and Gander, J. E. The 5-O-β-D-Galactofuranosyl-containing Exocellular Glycopeptide of Penicillium charlesii. Structural Characterization with Natural Abundance ^{13}C-Nuclear Magnetic Resonance Spectroscopy.

Pletcher, C. H., Lohmar, P. D. and Gander, J. E. Extracellular exo-β-D-Galactofuranosidase from Penicillium charlesii. Factors Influencing Stability.

Personnel Participating in Project

Research Assistants	Interval	Degree earned
Joanna Beachy	07/01/76-11/30/76	Ph.D.
Mark T. Johnson	07/01/76-07/31/76	Ph.D.
*Carol H. Pletcher	05/01/77-present	Ph.D. candidate
Cynthia J. Laybourn	07/01/77-12/31/77	M.S. candidate
Clifford J. Unkefer	09/16/76-06/30/77	M.S. candidate
Rodney Salo	07/01/76-11/30/76 05/01/77-05/31/77	M.S.

Technicians	
Faye Fang (Jr. Scientist)	07/01/75-08/31/77
Michael McGrath	07/01/75-09/15/75
Lora Hogy (Sr. Lab. Technician)	08/01/77-12/16/77
Doris Midgarden (Jr. Scientist)	12/28/77-6/14/78
Miscellaneous hourly help	

*Ms. Pletcher is working on this project; however she has a fellowship (American Association of University Women) which provides her stipend.

Legends to Figures

Fig. 1. Stability of galactofuranosidase in 5 min. incubations at varying temperatures. 50 µl of a DEAE fraction were incubated for 5 min at the indicated temperature, cooled to room temperature and then assayed.

Fig. 2. Inactivation of galactofuranosidase during an incubation at 60°C. 50 µl of a DEAE fraction were incubated for varying times at 60°C. Samples were removed, cooled to room temperature, and then assayed.

Fig. 3. Galactofuranosidase activity in the medium filtrate on Day 14. (——) 5 mmoles $(NH_4)_2HPO_4$ added on the indicated days, and (o——o) 150 mg glucose added on the indicated days. Aliquots were filtered on day 14, dialyzed, and assayed for 2 hr at 37° with 600 nmoles methyl-β-D-galactoside.

Fig. 4. Galactofuranosidase activity and pH or normal growth medium and $(NH_4)_2HPO_4$ modified growth medium. Galactofuranosidase activity in normal growth medium (o——o), galactofuranosidase activity in medium to which 5 mmoles $(NH_4)_2HPO_4$ was added on Day 6. pH of the normal growth medium (o——o); pH of medium to which 5 mmoles $(NH_4)_2HPO_4$ was added on Day 6.

Fig. 5a. The pH of normal growth medium and medium to which additional nitrogen-containing compounds were added on Day 3. The quantity of added bovine serum albumin ammonium carbonate and casein amino acids were 750 mg; 3 mMoles and 750 mg per 150 ml, respectively.

Fig. 5b. Galactofuranosidase activity in normal growth medium and in medium to which additional nitrogen was added on Day 3. Aliquots of medium were filtered, dialyzed, and assayed with 1.2 µmoles β-D-methylgalactofuranoside. The quantities of each nitrogen-containing substance is given in Fig. 5a.

Fig. 6. Galactofuranosidase activity and pH of Na_2CO_3 and NaOH modified growth medium. On Day 3 either 5N NaOH was added to adjust the pH to 4 or 3 mmoles of Na_2CO_3 was added to the culture. Aliquots of medium were filtered and the pH was measured; then the aliquots were dialyzed and assayed with 1.2 µmoles of β-D-methylgalactofuranoside.

Fig. 7. ^{13}C-Proton decoupled NMR spectrum of phosphogalactomannan (100 mg ml^{-1}) in H_2O (pH 7.0). The spectrum was taken on a Varian XL100 spectrometer at 38°C. Peaks labelled 1-5 have been assigned to the carbons of galactofuranosyl residues (peak 1, 108.0 ppm, carbon 1; peak 2, 82.4 ppm, carbons 2 and 4; peak 3, 77.5 ppm, carbon 3; peak 4, 76.6 ppm, carbon 5; peak 5, 62 ppm, carbon 6 (37).

Fig. 1

STABILITY OF GALACTOFURANOSIDASE IN 5 MIN.

INCUBATIONS AT VARYING TEMPERATURES

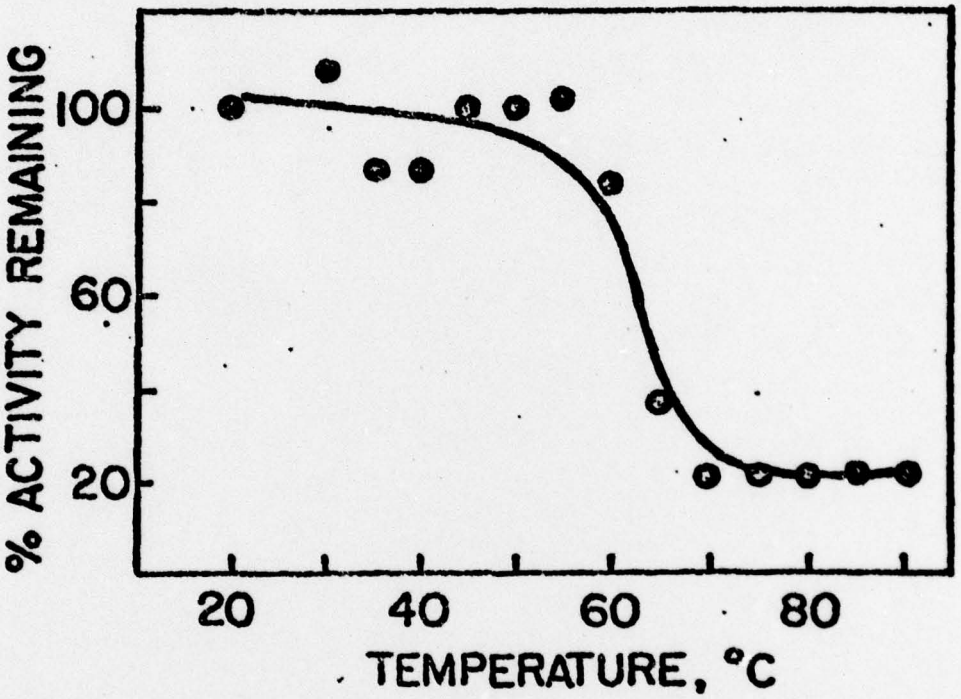


Fig. 2

INACTIVATION OF GALACTOFURANOSIDASE AT 60°C.

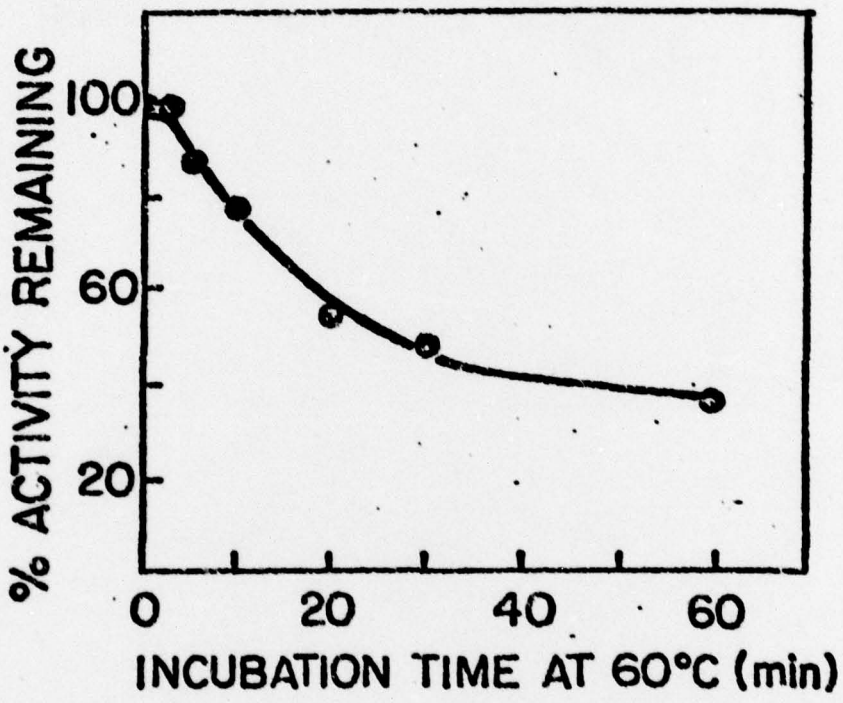
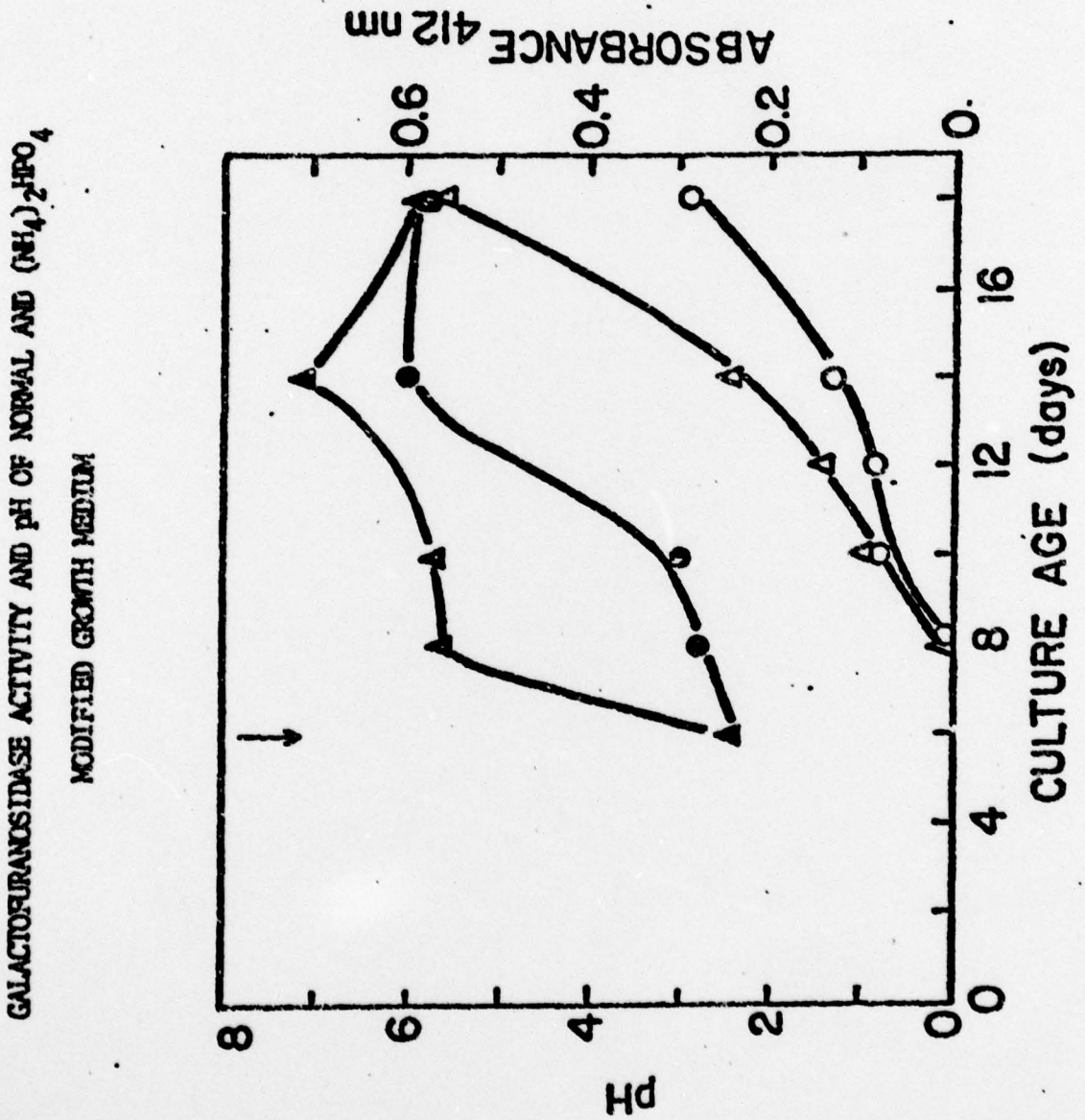


Fig. 3


GALACTOFURANOSIDASE ACTIVITY AND pH OF NORMAL AND $(NH_4)_2HPO_4$
MODIFIED GROWTH MEDIUM
pH
ABSORBANCE 412 nm
CULTURE AGE (days)
0
2
4
6
8
0.2
0.4
0.6
0
4
8
12
16

GALACTOFURANOSIDASE ACTIVITY IN CULTURE FILTRATES ON DAY 14

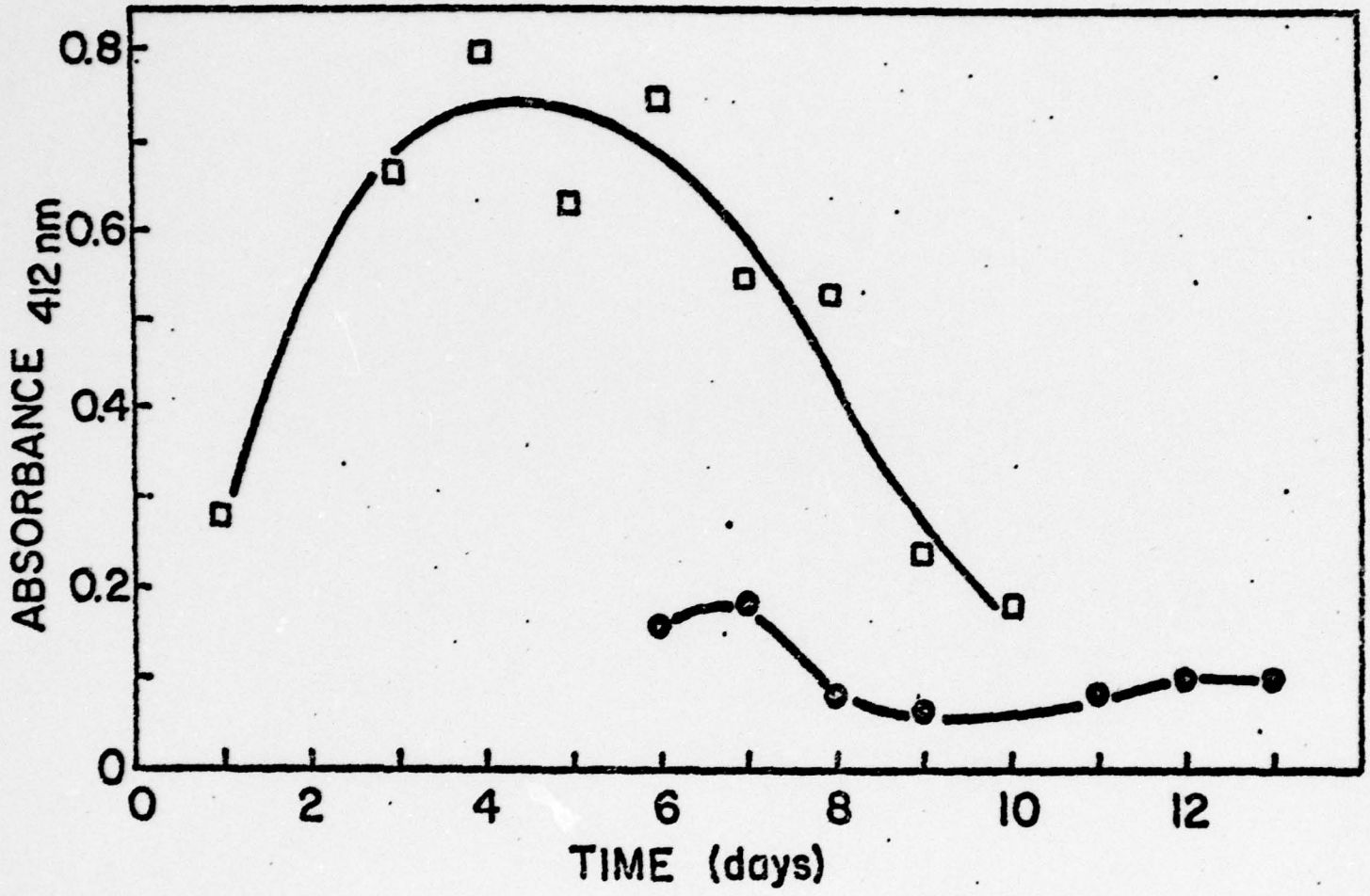


Fig. 4

Fig. 5a

INFLUENCE OF NITROGEN SOURCE ON THE pH OF THE MEDIUM

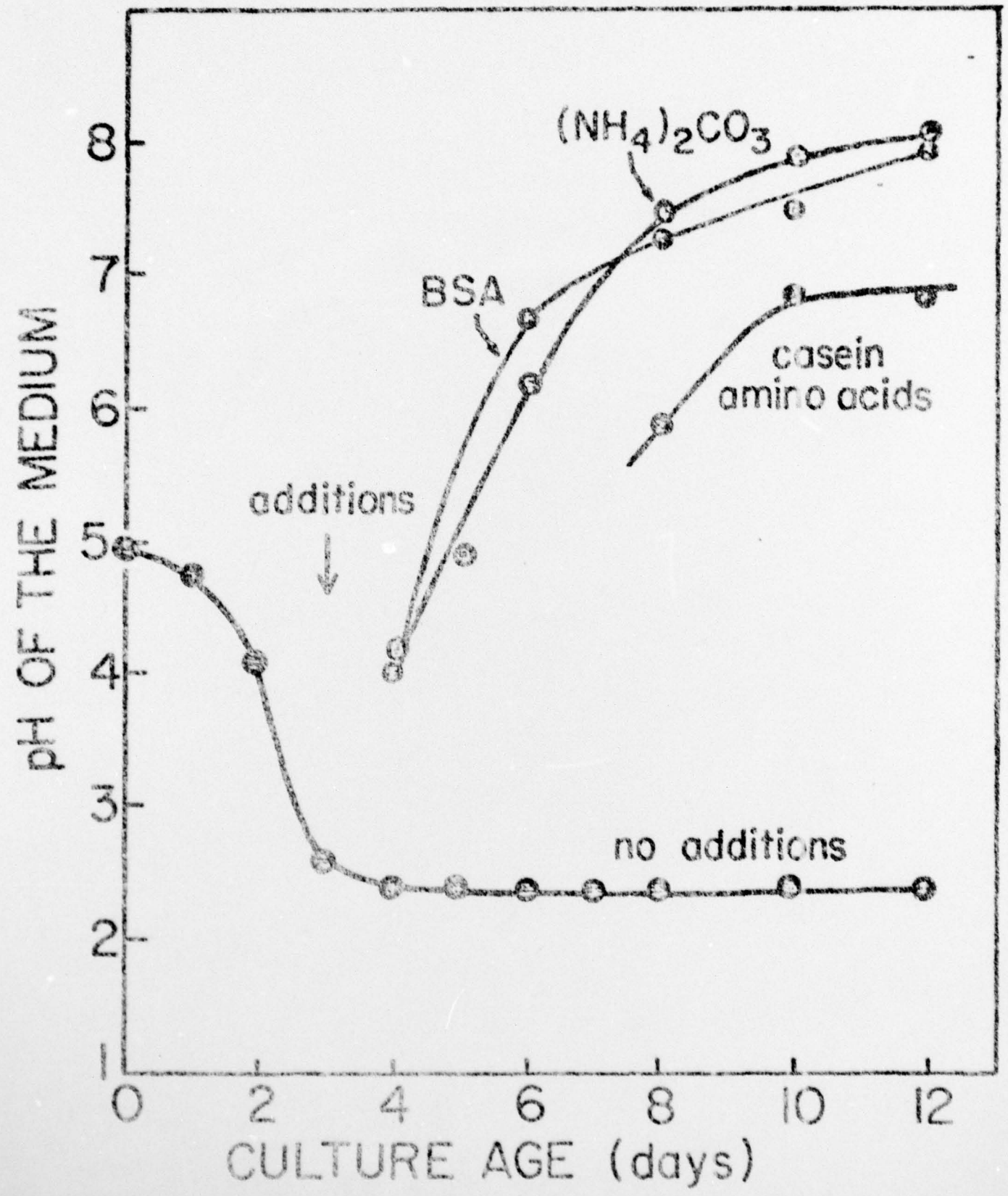
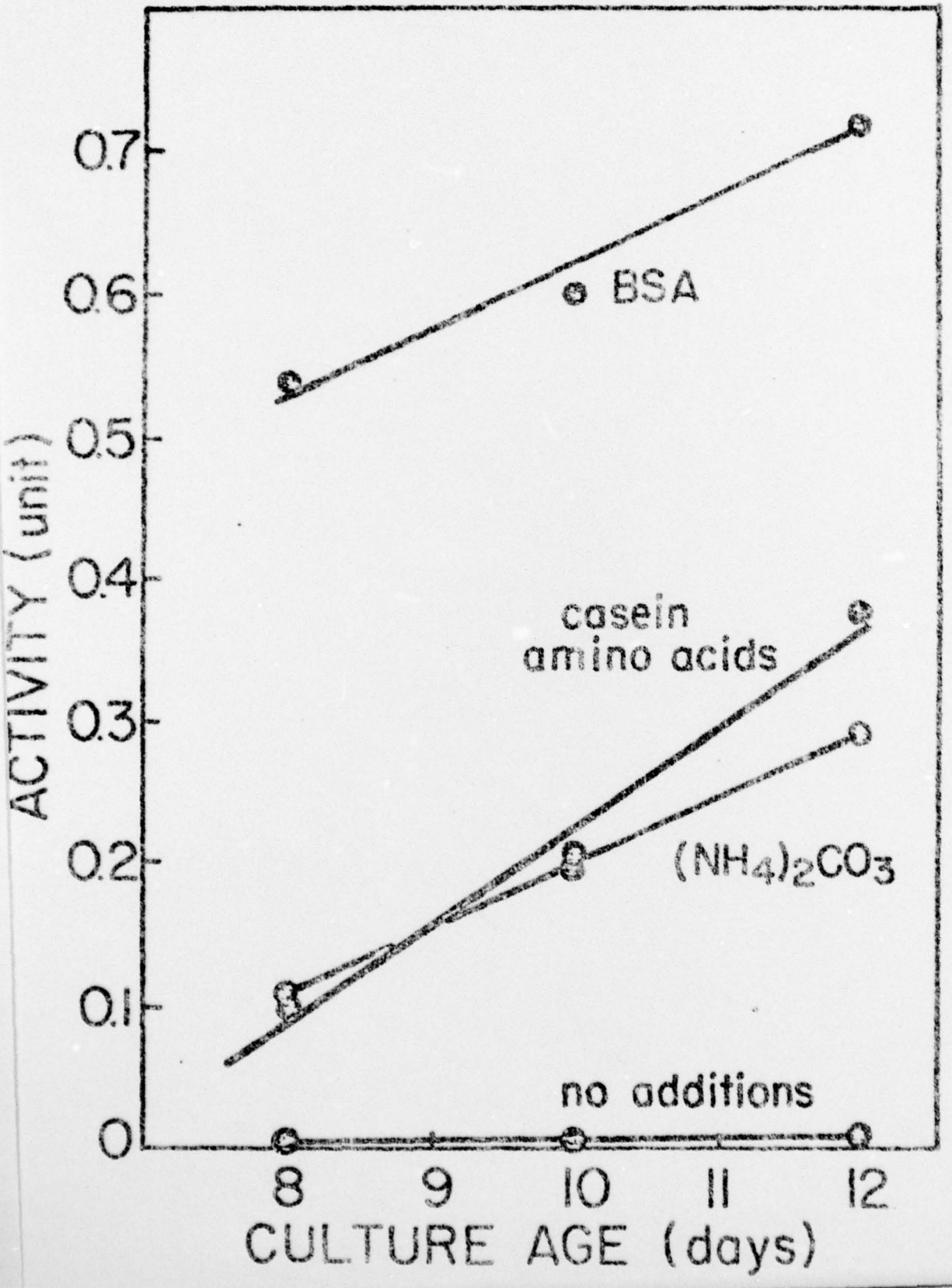
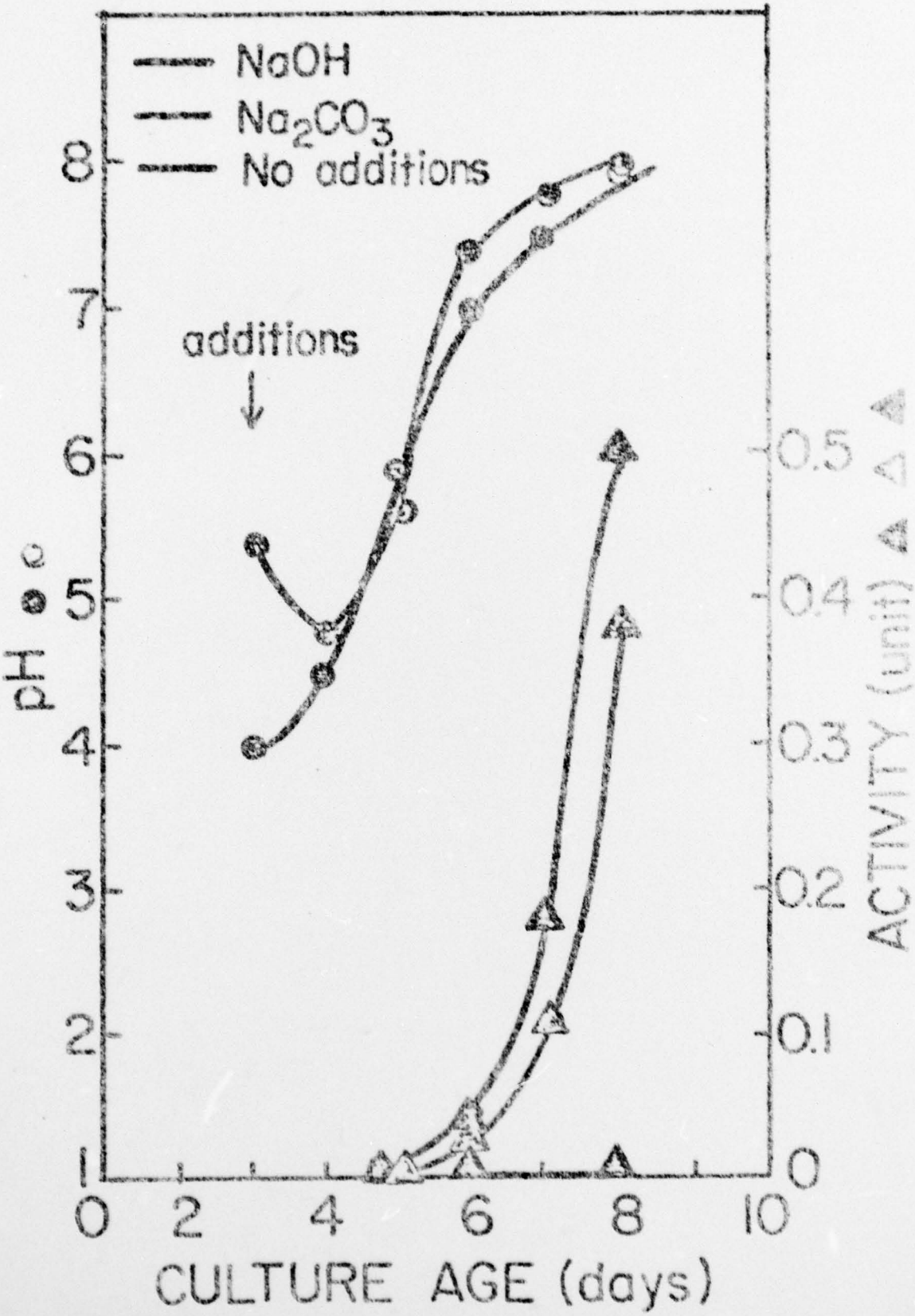


Fig. 5b

INFLUENCE OF NITROGEN SOURCE ON THE APPEARANCE OF GALACTOFURANOSIDASE



EFFECT OF pH ON THE APPEARANCE OF GALACTOFURANOSIDASE



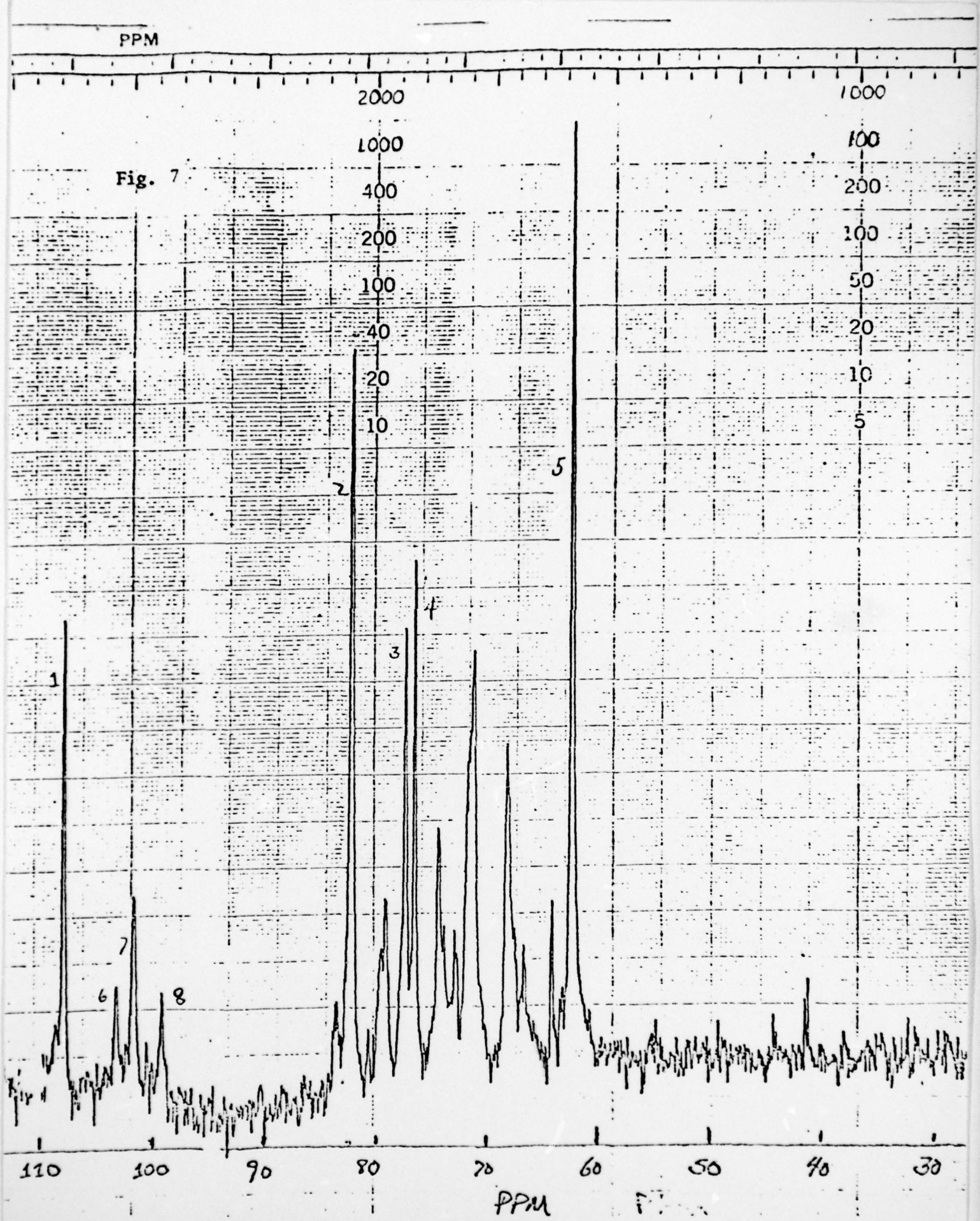

PPM
2000
1000
1000
400
200
100
40
20
10
100
200
100
50
20
10
5
1
2
3
4
5
6
7
8
110
100
90
80
70
60
50
40
30
PPM

Fig. 7